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Serum Exosomes Expressing CD9, CD63 and HER2 From Breast-Cancer Patients Decreased After Surgery of the Primary Tumor: A Potential Biomarker of Tumor Burden

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Abstract. *Background/Aim: Exosomes are extracellular vesicles produced by both normal and cancer cells. Previous research has demonstrated that circulating exosomes derived from cancer cells may create a niche for future metastasis, distant from the primary tumor. In the present report, circulating exosomes were captured and quantified based on exosome-surface proteins in pre- and post-operative serum of breast cancer patients, focusing on the exosome markers CD9 and CD63, as well as HER2, a therapeutic target for breast cancer. Materials and Methods: Eight breast cancer patients were recruited, and their pre- and post-operative serum samples were analyzed for CD63 and CD9; or CD9 and human epidermal growth factor receptor-2 (HER2), double-positive exosomes. An ExoCounter with antibody-conjugated beads was used to capture serum-derived exosomes. Sera from patients with tumors larger than 10 mm were used for analysis. The resected breast cancer was also histopathologically analyzed for the presence of HER2. Results: CD63 and CD9 double-positive serum exosomes and CD9 and HER2 double-positive serum exosomes decreased after surgery in breast-cancer patients whose tumors expressed HER2, as determined by histopathological analysis. Conclusion: Serum exosomes expressing CD9, CD63 and HER2 are candidate biomarkers of tumor burden in HER2-positive breast-cancer patients.*

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Key Words: Exosomes, cancer cells, breast cancer, CD63, CD9, HER2, serum, mastectomy, tumor burden, biomarker.

Exosomes are small extracellular vesicles surrounded by a lipid bilayer, approximately 30-150 nm in size, that are secreted by both normal and cancer cells and contain donor-cell-derived components both inside and on the surface (1, 2). Exosomes containing miRNA, mRNA and proteins, serve as mediators of intracellular communication (3-5). Cancer cells secrete exosomes into the tumor microenvironment, which are incorporated into cancer-associated cells (6). Exosomes secreted form cancer cells can also form a metastatic niche in distant organs (7, 8). Exosomes have also been found in various body fluids, including serum, urine, saliva, and tears (9-12). Exosomes in these body fluids are potential biomarkers for cancer diagnostics and treatment.

In the present study, we show that serum exosomes, expressing CD9, CD63 and human epidermal growth factor receptor-2 (HER2) on their surface and captured with specific antibodies, are reduced after surgery of the primary tumor, expressing HER2, of breast-cancer patients.

Materials and Methods

Clinical samples. The present study was approved by the Human Experimentation Committee of Kobe University Graduate School of Medicine and was conducted in accordance with the Helsinki Declaration of 1975. The present study was also approved by the Human Experimentation Committee of Kobe University (B210056). All patients have provided written informed consent. Patients with a primary breast-tumor diameter greater than 10 mm were eligible

Table I. *HER2 status in 8 patients. Tumor size and number of lymph node metastases are also described.*

Patient No.	HER2 IHC results	Tumor size (mm)	Lymph node metastasis
BC1	0	21×14	1
BC2	0	33×10	5
BC3	0	20×19	4
BC4	1+	11×10	0
BC5	2+	3×2 (DCIS 86×33)	0
BC6	2+	12×9	0
BC7	3+	43×29	0
BC8	3+	24×22	0

IHC: Immunohistochemistry; DCIS: ductal carcinoma *in situ*.

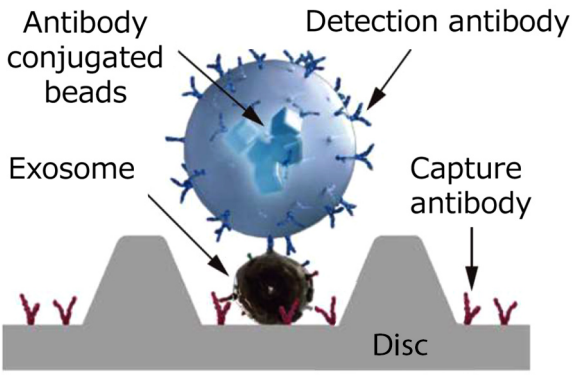

Figure 1. *Schematic of the ExoCounter. Illustration of exosomes labeled with nanobeads on a disc. Each exosome is separated into grooves on an optical disc coated with an antibody specific for exosomes. The exosomes are also recognized by a single bead labeled with another exosome-specific antibody. Theoretically, one exosome per bead can be counted.*

for the present study. Post-operative serum was collected between 11-41 days after the date of surgery (Table I).

All samples were stored at –80˚C and thawed on ice before exosome analyses. Serum samples were visually checked for hemolysis, and only hemolysis-free samples were used. HER2 staining was performed using a Roshe test kit (Roshe Diagnostics GmcH, Mannnheim, Germany) in the pathology laboratory of Kobe University Hospital. HER2- immunohistochemistry (IHC) results were obtained from clinical pathology (13).

Preparation of HER2 antibody-conjugated nanobeads. Biotin labeling of HER2 antibody (sc-33684, Santa Cruz Biotechnology, Dallas, TX, USA) was performed by binding to avidin-conjugated beads using the Biotin labeling kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan). Avidin-conjugated beads were washed with phosphate-buffered saline (PBS). Biotinylated HER2 antibody was conjugated to avidin-conjugated beads, washed with PBS which was replaced with HEPES buffer before use.

Exosome quantification with an ExoCounter. The quantification of exosomes by an ExoCounter was performed as described previously (14, 15) (Figure 1). The wells, in which discs with antibodies specific for CD63 or CD9 were conjugated, were washed three times with PBS-T (PBS containing 0.05% Tween20), injected with blocking solution (PBS-T containing 1% BSA), incubated at 37˚C for 30 min, and then washed three times with PBS-T. A total of 400 μl of blood were drawn from the patients by venipuncture. Then, 10 μl of serum were diluted in 40 μl of PBS and placed into each well, incubated at 37˚C for 2 h, and then washed three times with PBS-T. Then, 50 μl of specific antibody conjugated beads in buffer were applied to each well, incubated at 37˚C for 90 min, and washed three times with PBS-T. The wells were then washed with deionized water. Beads bound to exosomes on a disc were counted with the ExoCounter. The assay kit for the ExoCounter was purchased from JVC KENWOOD (BX-EAK1JA, BX-EAK2JA, Kanagawa, Japan) (15).

Statistical analysis. The Mann–Whitney *U*-test was used to determine the difference between the two groups. Statistical analyses were performed using Statcel 4 Software (OMS Publishing Inc., Tokyo, Japan).

Results

CD63 and CD9 double-positive exosomes in the serum of pre- and post-operative breast-cancer patients. CD63 and CD9 are exosome-marker proteins that are used for measuring the number of exosomes in serum. The number of exosomes that were positive for both CD63 and CD9 was measured with the ExoCounter. The number of CD63- and CD9-positive exosomes from the serum of post-operative breast-cancer patient was significantly reduced compared to pre-operative serum ($p<0.05$) as measured with the ExoCounter (Figure 2).

CD9 and HER2 double-positive serum exosomes decreased after mastectomy. HER2-positive (IHC 1+, 2+, 3+) breast-cancer patients had decreased CD9 and HER2 double-positive exosomes after mastectomy ($p<0.05$) as measured with the ExoCounter (Figure 3).

Discussion

Cancer cells secrete exosomes around the tumor and into the bloodstream and use them as mediators of intercellular communication (1, 3-5, 16, 17). Cancer-cell-derived exosomes also can form a niche in distant organs for future metastasis (7, 8, 18, 19). Thus, cancer-secreted exosomes could be used for diagnostic and prognostic biomarkers of various cancers (20).

The present study analyzed pre- and post-operative serum exosomes of breast-cancer patients and the pathology of breast-cancer samples. The number of CD63 and CD9 double-positive exosomes in serum was significantly decreased after mastectomy. Previous reports have shown that CD9- and CD63- positive exosomes are highly associated with breast-cancer metastasis (21). There are also reports that there is a

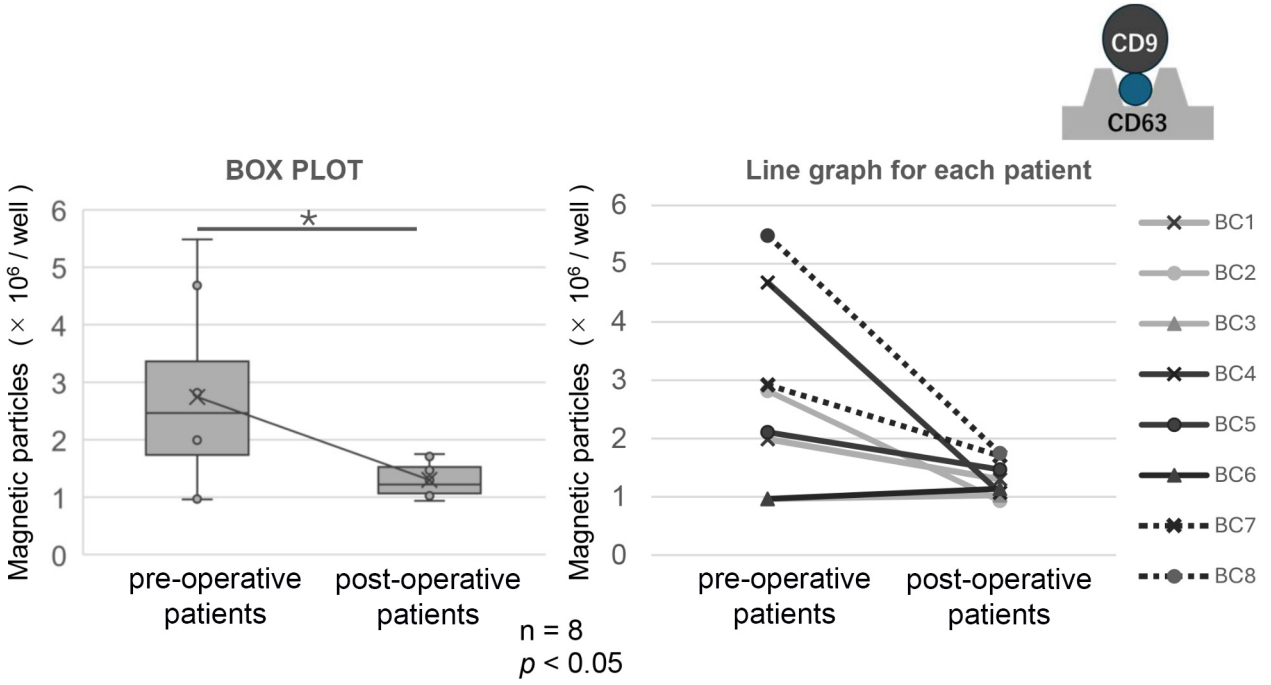

Figure 2. *Pre- and post-operative CD63 and CD9 double-positive serum exosome quantification. The left figure is displayed as a box-and-whisker diagram. The horizontal line represents the median. Box height represents the interquartile range, between the 75th and 25th percentile of individual fold-change. Data were statistically analyzed using the Mann-Whitney U-test (p<0.05). In addition, the right figure shows a line graph of pre- and post-operative changes for each patient.*

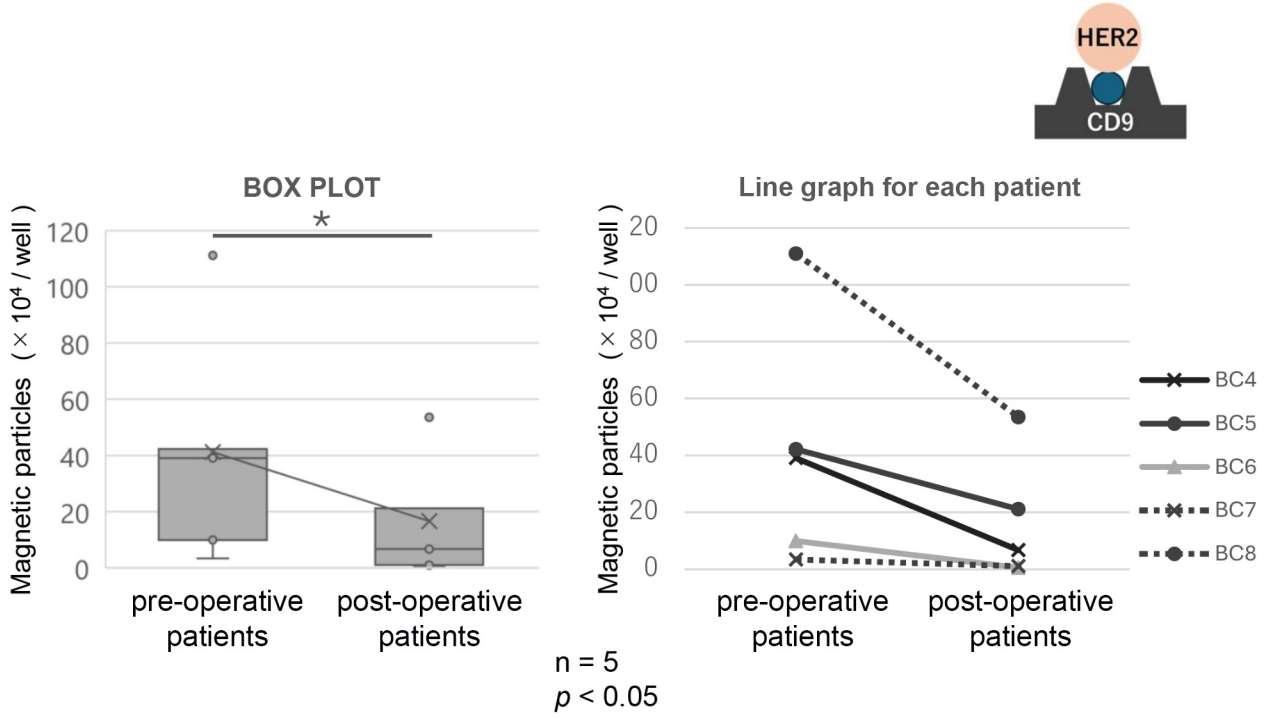

Figure 3. *Pre- and post-operative CD9 and HER2 double-positive serum exosome quantification. CD9 and HER2 double-positive exosomes in the serum of breast-cancer patients (n=5) were quantitated pre- and post-operatively. Data were statistically analyzed using the Mann-Whitney U-test (p<0.05).*

correlation between tumor volume and exosome volume (22), and we suggest that this is related to the postoperative decrease of CD9- and CD63- double-positive exosomes.

Breast-cancer patients expressing HER2 in their primary tumor, determined by histopathology, had decreased CD9 and HER2 double-positive exosomes after mastectomy. CD9 and HER2 double-positive exosome levels were unchanged pre and post-operatively in HER2-IHC-negative (o) breast-cancer samples.

We have reported that methionine restriction increases exosome secretion from cancer cells (23). This suggests that further research is needed on the effect of patient's nutritional status on serum exosome levels.

Recently, micro-residual disease (MRD) analyses have been performed using circulating tumor DNA (ctDNA) (24), and the detection of exosomes in serum may also be useful to detect early recurrence of HER2-positive (IHC 1+, 2+, 3+) breast cancer. In addition, more than 80% of primary breast cancers are HER2-positive (IHC 1+, 2+, 3+), making HER2 on the surface of exosomes a promising target for detecting breast cancer (25).

Analysis of the number of exosomes that are positive for both CD63 and CD9, and the number of exosomes that are positive for both CD9 and HER2, are thus candidates as biomarkers for breast-cancer tumor burden. Further research is necessary.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conception and design: SI, HT. Performed the experiments: SI. Sample collection: TK, MK, SI, MY, MO, YY, MM, SM, MB. Manuscript writing: SI and RMH. Approval of manuscript: All Authors.

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